

## Cell behaviour on bioactive polymeric coatings

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### Summary

Researchers are testing different treatments to induce modifications in both chemical composition and topography of implant surface, with the aim to ameliorate bone-to-implant contacts and hence improve osseointegration processes and biomechanical properties in the short and long term. Aim of the present research was the evaluation of MG63 osteoblast-like cells behaviour on polymeric coatings, electrosynthesized on titanium substrates, differently modified in order to improve implant performances both in terms of osseointegration and infection prevention. Cell viability data and scanning electron microscopy morphological observations were consistent with a good compatibility of modified electrosynthesized coatings and suggest the use of this procedure to produce new bioactive titanium coatings for implant surgery.

### Introduction

The performances of implanted materials rely on the nature of the interactions between biomaterials and cells (Boyan et al, 1996; Puleo et al, 1999). Different treatments have been performed to induce modifications in both chemical composition and topography of implant surface (Larsson et al, 1996) with the aim to ameliorate bone-to-implant contacts (Bigerelle et al, 2002), thus improving the osseointegration processes (Anselme et al, 2000).

Among the possible metal-implant surface modification methods, the electrochemical polymerization (ECP) represents a valuable strategy to develop polymeric coatings able to improve implant biocompatibility. The ECP provides the advantage of obtaining polymeric coatings directly onto metal substrates having different shape and size with a one-step procedure (Kwok-Keung et al, 1995; Cram et al, 2002; De Giglio et al, 2010).

Considering the polymers exploitable as biomedical devices, there are those obtained from acrylic monomers and, among these, hydrogels. The latter are polymeric three-dimensional networks able to retain a large amount of water and several studies were addressed to the development of various hydrogel networks for applications in drug release, tissue engineering, etc. (Montheard et al, 1992; Park et al, 1993; Ratner, 1993; Burdick et al, 2002).

The development of thin hydrogel coatings by ECP on metal device surfaces is therefore an interesting target in order to obtain metal biomaterials releasing biologi-

cally active molecules (antibiotics, growth factors, drugs, etc.) entrapped within the hydrogel layer during or post ECP. In this respect we have evaluated MG63 osteoblast-like cells behaviour on Poly-Hydroxy Ethyl MethAcrylate (pHEMA) coatings, electrosynthesized on titanium substrates, differently modified in order to ameliorate implant performances. In one case this change was addressed to enhance both mineralization and angiogenesis at the implant site: for this purpose HEMA was copolymerised with a phosphate-containing monomer, 2-Methacryloyl-Oxy-Ethyl-Phosphate (MOEP) and subsequently loaded with recombinant human Vascular Endothelial Growth Factor (rhVEGF). Moreover, since infection in implant surgery is a serious complication and a major cause of implant failure, ciprofloxacin (CIP)-modified pHEMA coatings have also been developed.

Adhesion and viability of MG63 osteoblast-like cells on the modified pHEMA coatings as well as the capability of both systems to entrap and release the growth factor (rhVEGF) or the antibiotic (CIP) respectively have been assessed.

## Materials and Methods

*Coating electrosynthesis:* Coatings were applied by ECP, in aqueous medium, of 0.1 M HEMA and 0.1 M HEMA copolymerised with 0.05M MOEP (both from Fluka, Sigma-Aldrich, Milan, Italy) on Ti sheets measuring 1cm<sup>2</sup>. ECP process has already been described (De Giglio *et al.*, 2009). Briefly, using cyclic voltammetry as electrochemical technique, with PAR 273 potentiostat-galvanostat (EG&G Princeton Applied Research, NY, USA), the potential was cycled between 0.0 V and -1.2 V (vs Ag/AgCl, KCl sat.) for 20 cycles (scan rate 100 mV/s).

*X-ray Photoelectron Spectroscopy (XPS) analysis:* XPS spectra were obtained with a ThermoVG Thetaprobe spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) equipped with a microspot monochromatised AlK $\alpha$  source. The AlK $\alpha$  line (1486.6 eV) was used throughout; the base pressure was 10<sup>-9</sup> mbar. Survey scans (binding energy range 0-1200 eV, FAT mode, pass energy = 150 eV) and detailed spectra (FAT mode, pass energy = 50 eV) were recorded. Data were analysed using the Advantage software package, consisting of a non-linear least-squares fitting program. The experimental points of the detailed spectra were fitted using Gaussian-Lorentzian peaks according to fitting models previously applied (De Giglio *et al.*, 2009).

*rhVEGF entrapment and release:* rhVEGF (ProSpec-Tany TechnoGene Ltd, Rehovot, Israel) was entrapped in the ECP p(HEMA-MOEP) films by dipping the coated Ti sheets into a PBS solution containing rhVEGF (50  $\mu$ g/mL) for 2 h. The sheets were then rinsed with PBS and dried under a nitrogen flux. To assess rhVEGF entrapment, coatings were incubated with anti-VEGF monoclonal antibody (Santa Cruz Biotechnology Inc., CA, USA) for 1 h at room temperature. A FITC-conjugated rabbit-antimouse IgG antibody (1:500, AlexaFluor®, Invitrogen, Milan, Italy) was added for 30 min. Fluorescent immunocomplex VEGF was visualised with an inverted confocal microscope (CLSM510, Zeiss, USA). rhVEGF release was assessed after incubation (48 h) of specimens in DMEM or PBS using a commercial enzyme immunoassay kit (human VEGF ELISA kit, DBA, Milan, Italy).

*Antibiotic entrapment and release:* Ciprofloxacin (CIP) was entrapped within hydrogel coatings after ECP by dipping titanium sheets in antibiotic solution 100 mg/mL

or 1000 mg/mL in NaCl 0.9%, for 2 h at 37°C. Finally the sheets were removed from the solution and rinsed with distilled water. CIP release from pHEMA hydrogel coatings was monitored in a physiological solution (NaCl 0.9%) at 37°C. An amount of the solution was drawn after 48 h and used for HPLC assay standard curve. The ciprofloxacin detection was carried out using a high-performance liquid chromatograph (Agilent Technologies Ltd, Santa Clara, CA, USA) Model 1100 and an UV-DAD (Agilent Technologies Ltd. mod. 1100) equipped with BIORAD software for signal analysis. The chromatographic separation was allowed by a Restek RP-C18 analytical column (5  $\mu$ m, 250 mm x 4.6 mm internal diameter) (Kamberi et al, 1998).

**Cell cultures:** MG-63 human osteoblast-like cells (ATCC, Rockville, MD, USA) were cultured in 5% CO<sub>2</sub> controlled atmosphere (T=37°C) in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% foetal bovine serum (FBS), 1% L-glutamine (Sigma), and 1% antibiotics (penicillin-streptomycin; Gibco-Ivitrogen, Milan, Italy). After thawing they were routinely split 1:10 every 2-3 days and used at the 4<sup>th</sup> passage. MG63 cells were detached using 0.25% trypsin in 1mM EDTA (Sigma) and plated in triplicate onto the ECP coated Ti materials in 24-well polystyrene tissue culture plates (TCPs) at a density of 1x10<sup>4</sup> cells/cm<sup>2</sup> and directly onto TCPs wells used as control. Cell behaviour was assessed at 48 h (on the basis of coating swelling time) and, on samples modified in order to improve mineralization, also at 21 days. Three different experiments were performed (n=9).

**MTT (3-dimethylthiazol-2,5-diphenyltetrazolium bromide) colorimetric assay:** After incubation (48 h and 21 days) the medium was removed; 200  $\mu$ L of MTT (Aldrich 135038, Sigma) solution (5 mg/mL in DMEM without phenol red) and 1.8 mL DMEM were added to all cell monolayers; the multi-well plates were incubated at 37°C for a further 4 h. After discarding the supernatants, the dark blue formazan crystals were dissolved by adding 2 mL of solvent (4% HCl 1N in isopropanol absolute) and quantified by spectrophotometry (Secomam, Anthelie light, version 3.8, Contardi, Italy) at 570 nm. The results are reported as percentage of TCPs cultures (controls).

**Scanning Electron Microscopy (SEM):** Specimens were observed with a Philips XL 20 scanning electron microscope using the secondary electron detector (FEI Italia SRL, Milan, Italy). For SEM analysis specimens were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), post-fixed in 1% osmium tetroxide, dehydrated in increasing ethanol concentrations, critical point dried, mounted on aluminium stubs and gold-sputtered.

**Osteocalcin mRNA:** After 21 days in culture total RNA was extracted (Trizol, Invitrogen, Milan, Italy). Reverse-transcription and real-time PCR of quantified RNA amounts from samples and controls were performed using the Super Script III Platinum Two Step qRT-PCR Kit with SYBR Green (Invitrogen, Milan, Italy). Aliquots of cDNA were amplified with specific primers for GAPDH (internal control) and osteocalcin. The reaction was run in an MJ Research PTC-200 Chromo 4 (GMI Inc. Ramsey, Minnesota, USA).

## Results

**Coating characterization:** Electrosynthesis of pHEMA and p(HEMA-MOEP) on the Ti substrates yielded thin, adherent coatings that displayed some differences in their

voltammograms. XPS analysis of the pHEMA coatings demonstrated only carbon and oxygen signals in the wide-scan spectra, while when MOEP copolymers were analysed phosphorus was also detected. Negatively charged phosphate groups on the surface of p(HEMA-MOEP) were obtained by an alkaline treatment. XPS analysis performed on hydrogel thin coatings dipped in a CIP solution evidenced the presence of fluorine, which is a typical marker element for XPS investigation of CIP molecule (data not shown).

*Entrapment and release of rhVEGF:* Fine, diffuse rhVEGF positivity was demonstrated on the homogeneous surface network of p(HEMA-MOEP)-coated Ti surfaces (data not shown). Different amounts of rhVEGF were detected by ELISA after 48 h incubation of rhVEGF-containing p(HEMA-MOEP) in PBS ( $660 \pm 40$  pg/mL) or DMEM ( $920 \pm 55$  pg/mL), respectively.

*CIP release detection:* HPLC has been employed to quantify CIP release from the electrosynthesised coatings. CIP amounts, released after 48 h of incubation, were of  $0.280 \pm 0.003$   $\mu\text{g/mL}$  for pHEMA loaded with 100  $\mu\text{g/mL}$  of CIP and of  $0.660 \pm 0.002$   $\mu\text{g/mL}$  when the loaded amount was of 1000  $\mu\text{g/mL}$  of CIP.

*Cell behaviour:* The MTT test documented good MG-63 cell viability at 48 h and 21 days (Figure 1). No significant differences in cell adhesion, morphology and viability were detected when 100 mg/mL or 1000 mg/mL of antibiotic were tested. At 48 h SEM examination of cells seeded onto the electrosynthesised coatings showed evident cell spreading, indicating good attachment to the substrate. Moreover, SEM images of MG63 underline that the presence and/or release of antibiotic do not affect cell behaviour (fig. 2). At 21 days most MG63 cells grown on pHEMA (fig. 3a) displayed an elongated morphology, whereas on p(HEMA-MOEP) they appeared more spread (fig. 3b), as activated osteoblasts. Osteocalcin mRNA expression 21 days into culture was not significantly different between the coatings (0.82 pHEMA and 0.63 pHEMA-MOEP, respectively) and was comparable to that of MG63 cells cultured on TCPs without osteogenic conditioning media (not shown).

## Discussion

Titanium, pure or alloyed with other elements, is a widely used material for biomedical applications, due to its corrosion resistance, excellent biocompatibility, high mechanical performance, and good workability. For the application in dental and orthopaedic fields, a key parameter to achieve implant osseointegration, in addition to the biomechanical factors related to its geometry and shape, is the kind of interaction between the implant and the surrounding biological tissues. Indeed, a key role is played by the topography and chemistry of the implant surface (Tache et al, 2004).

In this work, the biocompatibility of differently modified of p(HEMA) polymeric coatings electrosynthesized on titanium substrates has been experimented using MG63 human osteoblast-like cells as representative cell culture model (Rausch-Fanm et al, 2008).

The ECP of thin p(HEMA-MOEP) coatings was devoted to promote both mineralization, due to the presence of negatively charged functional groups on the polymeric surface, and angiogenesis by release of entrapped rhVEGF as a result of hydrogel swelling properties. Our data were consistent with a good compatibility p(HEMA-

MOEP) electrosynthesized coatings. At 21 days of culture, even though features of cell activation were detected, no significant differences in mRNA osteocalcin expression were evidenced between negatively charged p(HEMA-MOEP) and pHEMA coatings. This aspect was in contrast with the results on the *in vitro* calcification processes by means of Simulated Body Fluid (SBF) in which an improvement in mineralization related to the presence of suitable functional groups was found (data not shown). Therefore it could be argued that for *in vivo* mineral deposition the requirement of a specific microenvironment other than cell system is a major need.

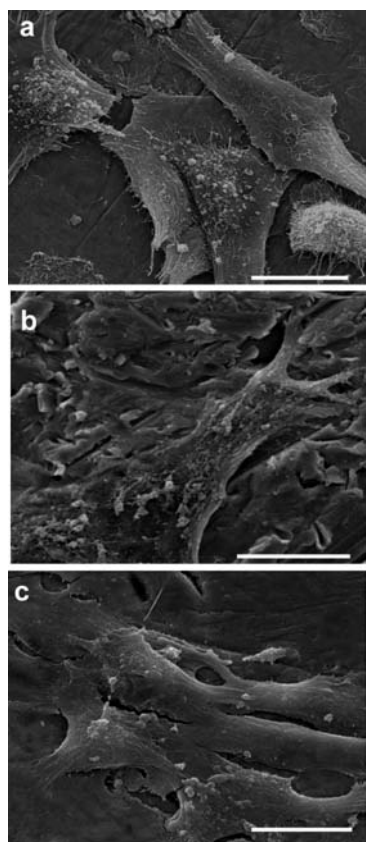
The loading of our coatings with antibiotic (CIP) was devoted to its release directly in the site of the implant, since implant-associated infections represent an occasional but serious problem in surgery. On CIP-loaded pHEMA coatings, cell behavior does not seem to be affected by the different CIP concentrations, as no significant differences in cell adhesion, morphology and viability were detected.

In conclusion, in this work the biocompatibility of new bioactive titanium implants coatings obtained by ECP of polyacrylic hydrogel films have been assessed. The obtained results suggest that these modified hydrogel coatings could represent an effective approach to favour bone-implant integration or to prevent bacterial infections without affecting osteoblast functions pertinent to new bone formation.

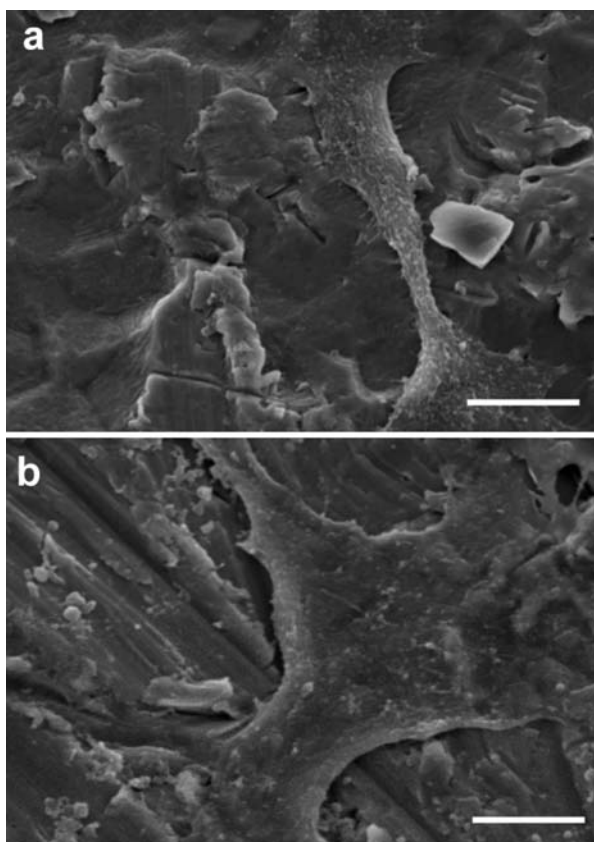
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**Figure 2** – SEM micrographs of MG63 cells cultured for 48h on pHEMA (a), p(HEMA-MOEP) (b) and CIP-loaded pHEMA (c) displaying a star-shaped morphology and adhesion to the substrate by means of filopodia. Figure scale bar 20 $\mu$ m.



**Figure 3** – SEM micrographs of MG63 cells cultured for 21d on pHEMA (a) and p(HEMA-MOEP): note the marked spreading of cells cultured on and p(HEMA-MOEP). Figure scale bar 10 $\mu$ m